Phytochemistry, 1970, Vol. 9, pp. 541 to 544. Pergamon Press. Printed in England.

A METHOD FOR THE SEPARATION AND DETECTION OF PLANT GLUCOSINOLASES (MYROSINASES)

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(*Received* 23 *June, in revised form* 29 *August* 1969)

Abstract—A method of detection of plant glucosinolases (myrosinases) after electrophoretic separation is described. Distinct differences in the isoenzyme pattern in different plants were observed, and in the expressed sap of some plant species at least four glucosinolase isoenzymes could be resolved.

INTRODUCTION

GLUCOSINOLATES, a class of glycoside of general structure, glucose—S—C(R)=N—O—$\bar{S}O_3$, are widely distributed among plants of the order Rhoeadales. They are degraded by plant enzymes (myrosinases) which at neutral pH values produce free glucose, sulphate and an isothiocyanate.

A recent paper,[1] which adequately summarizes past work, reports the isolation of myrosinase from mustard seed, and its separation by ion-exchange chromatography into two fractions, both of which liberated glucose as well as sulphate from the glucosinolate substrate (sinigrin). No separation of the sulphatase and thioglucosidase activities was obtained.

Vaughan *et al.*[2] examined myrosinase activity of extracts from several species of seed after starch gel electrophoresis. They detected the enzyme activity with sinigrin as a substrate by using a sensitive test for glucose which was allowed to diffuse on to a sensitized paper strip. The resolving power of this method of detection was limited therefore by the diffusion of substrate into the gel and of the product from the gel to the applied paper.

Since sulphate is also among the products of myrosinase action upon sinigrin, it was thought that regions of myrosinase activity in acrylamide gel electrophoretograms of plant proteins could be readily fixed in position and visualized by forming an insoluble sulphate in the gel. The results of the application of such a test to both seed and leaf preparations are now described. In agreement with our past practice and because organisms other than plants have been shown to contain myrosinase-like activity,[3–5] the term glucosinolase is preferred here; also the term glucosinolate is used to describe collectively the thioglucosides which act as substrates for the glucosinolases.

[1] I. TSURUO, M. YOSHIDA and T. HATA, *Agr. Biol. Chem.* **31**, 18 (1967).
[2] J. G. VAUGHAN, E. GORDON and D. ROBINSON, *Phytochem.* **7**, 1345 (1968).
[3] E. T. REESE, R. C. CLAPP and M. MANDELS, *Arch. Biochem Biophys.* **75**, 228 (1958).
[4] M. A. GREER, *Recent Prog. Horm. Res.* **18**, 187 (1962).
[5] D. B. MACGIBBON and R. M. ALLISON, *N.Z. J. Sci.* **11**, 440 (1968).

RESULTS AND DISCUSSION

Extracts from various tissues of a range of plants were subjected to disc gel electrophoresis, and the resulting gels treated to reveal the location of glucosinolase activity by sulphate precipitation. The results may be summarized as follows:

1. Extracts from leaves of all the glucosinolate-containing species examined demonstrated glucosinolase activity ranging from a single band to 4 or more isoenzymes in some species (Fig. 1).

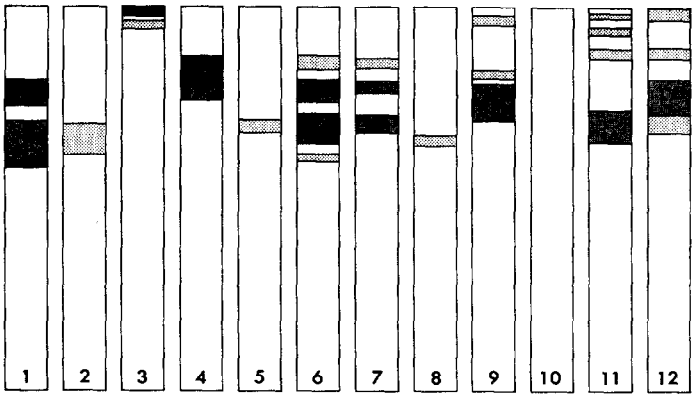

FIG. 1. PLANT GLUCOSINOLASES SEPARATED IN 8·6 PER CENT ACRYLAMIDE GELS.

Leaves

1. *Cardaria draba;*
2. *Lepidium sativum;*
3. *Raphanus sativus;*
4. *Cheiranthus cheiri;*
5. *Capsella bursa pastoris;*
6. *Brassica napus;*
7. *Brassica campestris.*

Seeds

8. *Lepidium sativum;*
9. *Raphanus sativus;*
10. *Cheiranthus cheiri;*
11. *Brassica napus;*
12. *Sinapis alba.*

Heavy shading denotes very strong bands.

2. Glucosinolase activity from the different species varied considerably as judged by the intensity of the developed bands and the time required for development after addition of the detecting reagent.

3. The pattern of glucosinolase isoenzymes varied within plant species depending on whether the source was leaf, stem, root or seed (Fig. 2).

The number of electrophoretically different glucosinolases and their identity and common occurrence in the different plant species and genera indicated in Fig. 1 warrants further investigation. Although we did not attempt to prove identity, visual comparison of the electrophoretograms from several *Brassica* species suggests that they possess certain isoenzymes in common, which may be of value in chemotaxonomy. As no separation of leaf glucosinolases appear to have been reported before, details of the methods used are presented in the Experimental.

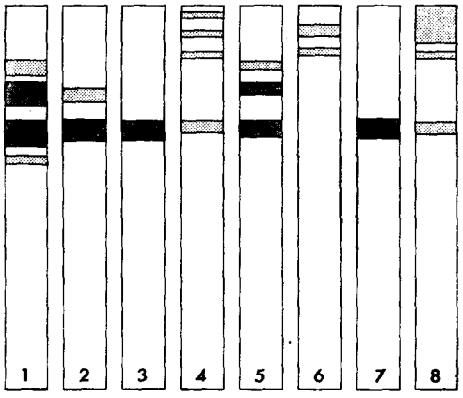

FIG. 2. GLUCOSINOLASES FROM DIFFERENT PARTS OF PLANTS.

Left–right *Brassica napus* (rape)
1. Leaf; 2. Petiole; 3. Root; 4. Seed.

Brassica campestris (turnip)
5. Leaf; 6. Petiole; 7. Root; 8. Seed.

EXPERIMENTAL

Gel Electrophoresis

The disc acrylamide gel system of electrophoresis as described by Davis[6] was used for separating the enzymes, using a concentration of 8·6 per cent acrylamide in the running gel. Electrophoresis was carried out for 2 hr at 3 mA per gel.

Sample Preparation

The most satisfactory method of sample preparation was to freeze several leaves for a few min at −10°, then remove and squeeze with the aid of a manual juice extractor about 2 ml of sap into a vial containing 0·8 g sucrose, 0·05 ml mercaptoethanol and 0·1 ml of the stock 'tris' buffer described by Davis.[6] It was preferable to express the sap first into the well of the juice extractor before placing it in the vial so that the mercaptoethanol was quickly diluted. Seed samples were prepared by stirring 20 mg of finely ground seed into 2 ml water containing 0·05 ml of mercaptoethanol and 0·1 ml of stock "tris" buffer. After filtering through glass-wool, sucrose was added to give a concentration similar to that of the leaf samples. Aliquots of 0·025 ml were applied to the gels with the aid of a syringe. Although activity remained in samples stored a day or two at room temperature, better definition of the separated bands was obtained from fresh samples. Storage at −10° tended to destroy glucosinolase activity.

Detection of Activity

The method used for detection of the glucosinolases depends primarily on the liberation of sulphate to form insoluble $BaSO_4$ bands when sinigrin is acted upon by a glucosinolase in the presence of $BaCl_2$. To a lesser extent the bands are formed by an insoluble hydrolysis product other than sulphate, probably sulphur.

Gels removed from their tubes by rimming under water were placed in small test-tubes containing an aqueous solution of sinigrin, 5 mg/ml, $BaCl_2$, 10 mg/ml, and ascorbic acid, 0·003 M. HOAc was added to give a solution with a concentration of 1·7 N (4 drops per tube). Each tube was covered then upturned several times to mix the acid. Development of white bands in the regions of glucosinolase activity took from several minutes up to 16 hr, depending on the enzymic activity and the temperature. Occasionally a faint bluish white band, clearly different in appearance from the glucosinolase bands, developed in gels of separated leaf proteins. This band was not due to glucosinolase as it did not require sinigrin for development.

The validity of the $BaSO_4$ method for the detection of glucosinolase activity was checked by segmenting gels of separated white mustard seed (*Sinapis alba*), rape seed and leaf, then testing each segment for its ability to release glucose and mustard oil from solutions of glucosinolate. Each sample was electrophoretically separated in triplicate gels. $BaSO_4$ bands were developed in one gel of each sample to act as markers. After

[6] BARUCH J. DAVIS, *Ann. N.Y. Acad. Sci.* **121** (2), 404 (1964).

the position of the bands had been determined in the marker gels by measurement, the other two gels of each sample were rimmed from their tubes and sliced into segments with a razor blade. Each gel was sliced into twelve segments against a rule such that the segments corresponding to the barium bands could be noted. Segments were tested for glucose liberation by placing each in 0·25 ml of an aqueous solution containing sinigrin, 10 mg/ml, ascorbic acid, 0·003 M, and HOAc, 1 N. After standing overnight, 20-μl spots of each solution were applied along the origin of a sheet of Whatman No. 1 chromatography paper along with standards of glucose and sinigrin. The chromatograms were developed for 24 hr by descent with the upper layer of *n*-butanol–ethanol–water (4:1:4) as the mobile phase. Glucose was detected with the $AgNO_3$ reagent.[7]

Gel segments were tested for liberation of mustard oil by placing each in 0·25 ml of an aqeuous solution of *Reseda luteola* (a rich source of glucobarbarin) prepared from a hot methanol extract of leaves.[8] After standing overnight, 20-μl spots were applied along the origin of an 8 in. square of Whatman No. 1 chromatography paper along with a 5-phenyl-2-oxazolidinethione standard. The chromatograms were developed by ascent using the upper layer of benzene–methanol–water (2:1:1) as the mobile phase. 5-Phenyl-2-oxazolidinethione was detected using iodine azide reagent,[9] with a starch spray for fixing the background. Of the twelve segments from each gel, glucose and mustard oil were shown to be released only by the segments corresponding to the $BaSO_4$ bands.

Photographic records of the separated isoenzymes were obtained by positioning the gels horizontally on a grooved strip of washing glass inside a flat-bottomed glass dish containing water. Two strips of wood served to hold the dish off the bench so that with safe-light illumination a piece of photographic printing paper could be held against the under side of the dish. After exposing for 10 sec under a 60 W incandescent bulb about 4 ft above, the prints were developed and fixed in the normal way.

[7] W. E. TREVELYAN, D. P. PROCTER and J. S. HARRISON, *Nature* **166**, 444 (1950).
[8] B. A. TAPPER and D. B. MACGIBBON, *Phytochem.* **6**, 749 (1967).
[9] F. FIEGL, *Spot Tests in Organic Analysis*, 5th ed., p. 88, Elsevier, Amsterdam (1956).